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Myofibrillar M-band proteins in rat skeletal muscles during development

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Summary. The distribution of three myofibrillar M-band proteins, myomesin, M-protein and the muscle isoform of creatine kinase, was investigated with immunocytochemical techniques in skeletal muscles of embryonic, fetal, newborn and four-week-old rats. Furthermore, muscles of newborn rats were denervated and examined at four weeks of age. In embryos, myomesin was present in all myotome muscle fibres of the somites, whereas M-protein was detected only in a small proportion of the myotome muscle fibres and muscle creatine kinase was not detected at all. In fetal and newborn muscles, all fibres contained all three M-band proteins. At four weeks of age, when fibre types (type 1 or slow twitch fibres and type 2 or fast twitch fibres) were clearly discernable, the pattern was changed. Myomesin and muscle creatine kinase were still observed in all fibres, whereas M-protein was present only in type 2 fibres. On the other hand, in muscle fibres denervated at birth all three M-band proteins were still detected. Our results suggest 1) that during the initial stages of myofibrillogenesis expression and incorporation of myomesin into the M-band precede that of M-protein and muscle creatine kinase; 2) that expression and incorporation of all three M-band proteins during fetal development is nerve independent and non coordinated to the expression of different forms of myosin heavy chains, and 3) that the suppression of M-protein synthesis during postnatal development is nerve dependent and reflects the maturation of slow twitch motor units.

Introduction

Three unequivocal M-band proteins have so far been identified, MM-creatine kinase (MM-CK, the dimeric form of the muscle isoenzyme of creatine kinase, MCK Mr 43000), M-protein (Mr 165000) and myomesin (Mr 185000). MM-CK is an enzyme involved in the energy metabolism of muscle cells. Most of the MM-CK is soluble, only a small fraction being firmly bound to myofibrils in the M-band (Turner et al. 1973; Wallimann et al. 1975, 1977a, b, 1978). From immunohistochemical and electron microscopic data, it has been proposed that MM-CK is involved in the formation of the M4,4′ bridges interlinking the thick filaments in register (Strehler et al. 1983; Wallimann et al. 1983; Wallimann and Eppenberger 1985). M-protein was first identified by Masaki and Takaiti in 1974 and has been proposed by Woodhead and Lowey (1982) to be related to the so called M-filaments which run parallel to the thick filaments and are supposed to interlink the M-bridges (Knappeis and Carlsen 1968; Luther and Squire 1978). In an immunoultrastructural study, polyclonal antibodies presumed to be against M-protein (at that time also called myomesin; Eppenberger et al. 1981) were shown to label the M-band along its whole length and at the peripheral set of M-bridges (Strehler et al. 1983). The polyclonal antisera used in that study (Strehler et al. 1983) reacted with both an M-band protein of Mr 165000 and one of Mr 185000. The latter is a distinct M-band protein now identified as myomesin (Grove et al. 1984). Therefore it is not yet clear whether M-protein and myomesin are differentially distributed within the M-band (Bähler et al. 1985; Thornell et al. 1986).

The purpose of the present study was to examine the composition of the M-band in rat skeletal muscle fibres at different stages of development and maturation using antibodies against myomesin, M-protein and MM-CK. Somites of the skeletal muscle myotome are present at 14 days of gestation. Muscle anlage with primary and secondary generation of myotubes are seen at late fetal stages as well as at birth. At this stage rat skeletal muscle fibres are polyneuronally innervated (Close 1964, 1972; Drachman and Johnson 1973; Riley 1977). At four weeks of age the poly-neuronal innervation has been eliminated (Lyons et al. 1983) and muscle fibre types are present containing the adult types of slow and fast myosin heavy chains (Butler-Browne and Whalen 1984; Gambke and Rubenstein 1984; Dhoot 1986; Narusawa

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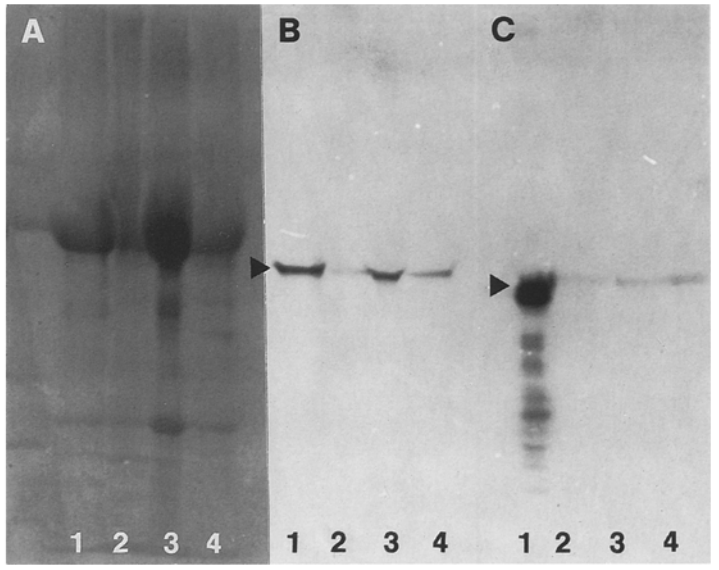

Fig. 1 A–C. Cross-reactivity and specificity of monoclonal antibodies against myomesin and M-band protein from chicken with myofibrils from rat skeletal and cardiac muscle. **A** Myofibrils from chicken pectoralis muscle (*1*); adult rat cardiac (*2*); rat soleus (*3*); and rat gastrocnemius muscle (*4*), separated by PAGE in 5% polycrylamide in the presence of SDS, electrophoretically transferred to PVDF membranes (immobilon, Millipore) and stained for protein with Ponceau-red. Molecular weight standards in kDa to the left. Note the predominance of myosin heavy chain protein at 200 kDa in this section of the gel. **B** Parallel blot as shown in **A** but stained with undiluted hybridoma supernatant containing monoclonal antibody B4 against chicken myomesin (*arrowhead at position of 185 kDa*) followed by incubation with 1:1000 diluted HRPO-conjugated second antibody and staining for peroxidase. Note that single bands in chicken as well as in rat muscles are obtained with B4 antibody. **C** Immunostaining of similar blot as shown in **B**, and by 1:100 diluted ascites fluid containing monoclonal anti-chicken M-band protein antibody A6, followed by 1:1000 diluted HRPO-conjugate second antibody and staining for peroxidase. Note the strong signal obtained with A6 antibody with the homologous chicken M-band protein (at 165 kDa, arrowhead) as well as with some degradation products of M-band protein generated during isolation of myofibrils (Grove et al. 1984). Single, but weak bands were obtained with both skeletal muscles and cardiac muscle from rat. However, as expected from histochemistry, myofibrils from soleus muscle had to be overloaded (lane 3 in A) in order to demonstrate the presence of some M-band protein in this muscle (compare with Fig. 5). Overall this results demonstrate that the anti-chicken antibodies used here (Grove et al. 1984) do specifically cross-react with skeletal and cardiac muscle from rat, although to a lower extent as compared with the homologous proteins from chicken

et al. 1987), and showing adult electrophysiological and functional characteristics (Close 1972; Brown and Booth 1983). As the process of postnatal muscle fibre diversification into fibre types is influenced by the motor neuronal activity, we also investigated whether denervation at birth would affect the M-band composition. Preliminary results of this study have been presented in Thornell et al. (1990).

Materials and methods

Pregnant, newborn (< 8 h post portum, 5–7 g) and four-week-old (90–100 g) Sprague-Dawley rats were used. The pregnancy was interrupted at different fetal gestation ages of 14, 16 and 18 days. The rats were anesthetized with Mebumal and the fetuses were collected and individually rapidly frozen in one block as described below. The newborn rats were anesthetized on ice and then decapitated (Fairfield 1948). The four-week-old rats were anesthetized with Mebumal. The soleus (SOL) and extensor digitorum longus (EDL) muscles on both hind legs were removed. In general, muscles from one side of the animal were used for histochemistry and the corresponding muscles from the other side were prepared for electron microscopy (Carlsson and Thornell 1987). For immuno- and enzyme-histochemistry blocks of skeletal muscle tissue were composed either of cross sections of the whole lower part of the leg from fetal rats or of SOL and EDL from newborn or four-week-old rats. The muscles were dissected and placed together either longitudinally or horizontally in a drop of the embedding medium (O.C.T. Compound, Miles Laboratories, Naperville, Illinois). All blocks were rapidly frozen by immersion in isopentane precooled with liquid nitrogen.

For analysis of the effect of denervation on the expression of M-band proteins, a small incision on the back of the thigh of the anesthetized newborn rat was made and segments (approximately 5 mm) of the sciatic nerve were removed. Thereafter the wound was sutured and sprayed with Nobecutan (Bofors Pharmaceutical Co., Södertälje, Sweden) (Bergh et al. 1978). The rats were warmed up and brought back to their mothers. These rats were examined at an age of four weeks and treated as the four-week-old normal rats.

Serial sections about 5 and 10 μm thick were cut in a Leitz (Wetzlar, FRG) cryostat and air dried. In order to obtain thin (0.5 to 1 μm) cryosections, legs of newborn rats and SOL of four-week-old rats were attached to toothpicks and chemically fixed either with 2% paraformaldehyde + 0.2% glutaraldehyde in relaxing buffer (0.1 *M* KCl, 5 mM EDTA, 0–1.0 m*M* EGTA at pH 7.0) for 2–4 h at 4° C or with 2.5% glutaraldehyde and Triton X-100 in relaxing buffer for 30 min at 4° C. Cryo-sectioning was performed on an Ultracut E fitted with a Cryokit FC4 (Reichert-Jung, Vienna, Austria) according to Tokuyasu (1980) or on an LKB Ultrotome III equipped with a Cryokit (LKB, Bromma, Sweden) according to the method described by Sjöström et al. (1973) with some modifications (Thornell et al. 1986).

Serial sections were stained for myofibrillar ATPase (Ca^{2+} activated myosin adenosine triphosphatase EC 3.6.1.3) at pH 9.4 or

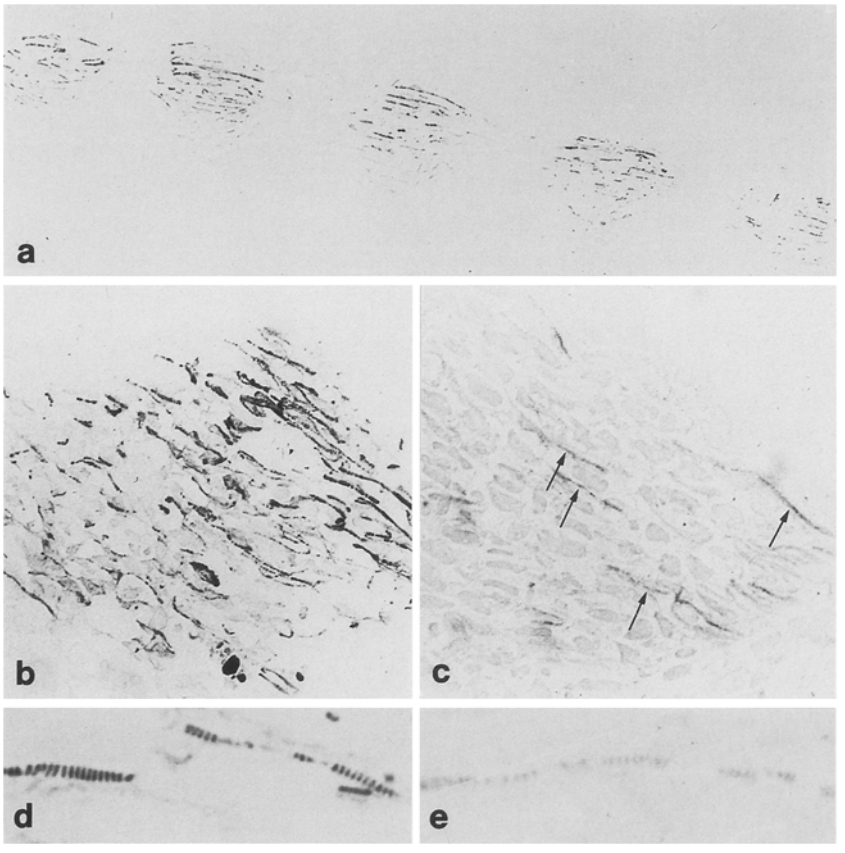

Fig. 2a–e. Serial sections of 14-day-old rat fetus showing somites or parts of somites stained with mAbs against myomesin **a, b, d** and M-protein **c, e** by the PAP technique. In **a** five somites are seen with myotome muscle fibres clearly apparent. In **b** many myotome muscle fibres are stained, whereas in **c** only some stained myotome myofibrils (*arrows*) are seen. In **d** and **e**, representing higher magnifications of **b** and **c** respectively, myofibrillar striations are apparent. **a** ×125; **b–c** ×640; **d–e** ×1,600

after an initial preincubation in a sodium barbital acetate buffer at pH 4.2 or 4.6 according to Brooke and Kaiser (1970). The sections were examined using a Leitz Dialux microscope.

MAbs against myomesin, Mr 185000, and M-protein, Mr 165000, from chicken pectoral muscle were used in the present study. Their preparation and characterization in chicken muscles have been described in detail (Grove et al. 1984, 1985). To determine the specificity of the mAbs on rat muscles, immunoblots of extracts from rat skeletal and rat heart ventricular muscles were performed. Procedures described in detail previously were followed (Grove et al. 1989). Anti-myomesin mAb (B4) recognized a protein of Mr 185000 and the anti-M-protein mAb (A6) a protein of 165000 in rat skeletal muscles as well as in the rat heart (Fig. 1), thus i.e., rat muscle contain isoforms of M-protein and myomesin which are very similar to those in chicken muscles. Goat antiserum against human MM-CK was obtained from Merck (Darmstadt, FRG). Rabbit and goat normal sera served as controls. Fluorescein-conjugated rabbit or sheep anti-goat IgG, rabbit anti-mouse IgG and mouse PAP complex were purchased from Dakopatts (Copenhagen, Denmark).

Cryostat sections in series with those used for histochemical staining for ATPase activity were used for immunohistochemistry. After a short rinse in phosphate-buffered saline (PBS) the sections were incubated at room temperature for 15 to 30 min with a drop of a solution containing specific antiserum or control serum diluted 50-fold in PBS followed by several changes of PBS. The sections were then incubated in the same way with either the appropriate fluorescent-labelled secondary antibody (diluted 1:40 to 1:100 in PBS) or with mouse PAP complex (1:50 in PBS with 0.1% BSA). In the latter case diaminobenzidine (0.1% in 0.05 *M Tris*, pH 7.6) plus 0.03% H_2O_2 were added for 10 min. After extensive washing in PBS for 30–60 minutes with several changes of buffer, the sections were mounted in glycerol/PBS 2:1 or in Mowiol 4–88, (Hoechst, Frankfurt, FRG), pH 8.5.

For immunolocalization of M-band bound MM-CK, the SOL was dissected from newborn and four-week-old rats and gently teased apart with syringe needles in relaxing buffer. After homogenization using a Polytron mixer (Kinematica AG, Luzern, Switzerland), the myofibrils were washed three times in the same buffer. Indirect immunofluorescent staining was performed as described earlier (Wallimann et al. 1977a).

For dual labelling, sections were either first incubated with mAbs against M-protein followed by tetramethylrhodamine-isothiocyanate (TRITC)-coupled rabbit anti-mouse antiserum and then with goat polyclonal antibodies against MM-CK followed by fluorescein isothiocyanate (FITC)-coupled rabbit anti-goat serum or vice versa. In some experiments both primary antibodies were applied simultaneously followed by the labelled secondary antibodies. Corresponding control sections were incubated with each antibody followed by each secondary antibody. No cross-reactivity of the secondary antibodies with the non-homologous IgG was observed. The sections were examined using a Leitz (Wetzlar, FRG) Orthoplane microscope equipped with epifluorescence optics. For fluorescein fluorescence, a selective excitation filter (BP 485/20 nm) was used in combination with a narrow band barrier filter (520–560 nm) in order to prevent rhodamine "bleeding through" in dual labelled specimens. Selective rhodamine fluorescence was observed using a BP 546/12 nm excitation filter and an LP 590 nm barrier filter.

Results

Somites

The somites were distinctly apparent in 14-day-old embryos in sections stained for myofibrillar ATPase at pH 9.4 (not illustrated) as well as in sections stained with mAb against myomesin (Fig. 2a). In the latter sections,

viewed at higher magnification, long, slender, striated myofibrils were clearly distinguished (Fig. 2b, d). In serial sections stained with mAbs against M-protein the somites were hardly discernable with only some myofibrils being stained (Fig. 2c). At high magnification some stained striations were apparent (Fig. 2e). Antibodies against MM-CK gave only a very faint cytoplasmic labelling of the somites (not shown).

Primary and secondary generation fibres

Antibodies against myomesin, M-protein and MM-CK stained all myotubes in both SOL and EDL muscles from 16, 18 days fetuses and primary and secondary fibres in newborn rats (only SOL of newborn rat shown, Fig. 3a–c). In longitudinal sections fluorescent cross-striations at the M-band level were apparent along the whole length of both myotubes and primary and secondary generation of fibres.

As MM-CK is present in both a soluble and a myofibrillar bound form isolated myofibrils from newborn rats SOL were washed and then stained for MM-CK. In such washed myofibrils distinct staining of the myofibrillar M-band was seen (Fig. 4).

Muscle fibre types

In cross section EDL and SOL muscle of four-week-old rats, individual fibres could be typed on the basis of ATPase activity at different pH level (Fig. 5c, d, g, h). All fibres in both muscles showed staining with anti-myomesin mAb (Fig. 5a, e) whereas only a portion of the fibres reacted with antibodies against M-protein (Fig. 5b, f). When the immunostained sections of both muscles were compared with serial sections stained by enzyme histochemistry (Fig. 5c, d, g, h), it became apparent that the fibres positive for M-protein were type 2 fibres (strong ATPase activity at pH 9.4 and low at pH 4.2), whereas type 1 fibres (low ATPase activity at pH 9.4 and strong activity at ph 4.2) were not stained. However, there were variable M-protein staining among the type 2 fibres which could be related to the presence of subtypes as 2A, 2C and 2X or 2D (Schiaffino et al. 1989; Termin et al. 1989; Gorza 1990). Type 2B fibres (high activity at pH 9.4 and 4.6, low activity at pH 4.2) showed the strongest M-protein staining and type 2A, 2C and 2D or 2X fibres showed various degrees of anti-M-protein labelling.

In cross sections of EDL and SOL muscles labelled with antibodies against MM-CK, staining was seen in all fibres with stronger staining in type 2 fibres (data not shown). In longitudinal sections similarly stained a striated pattern was often difficult to resolve because of high overall staining. However, distinct anti-MM-CK striations were readily apparent in washed myofibrils (Fig. 4d) as well as in semithin cryosections (Fig. 6d). Coincident staining patterns obtained with anti-myomesin and anti-MM-CK in dual labelling experiments confirmed the M-band localization of these proteins

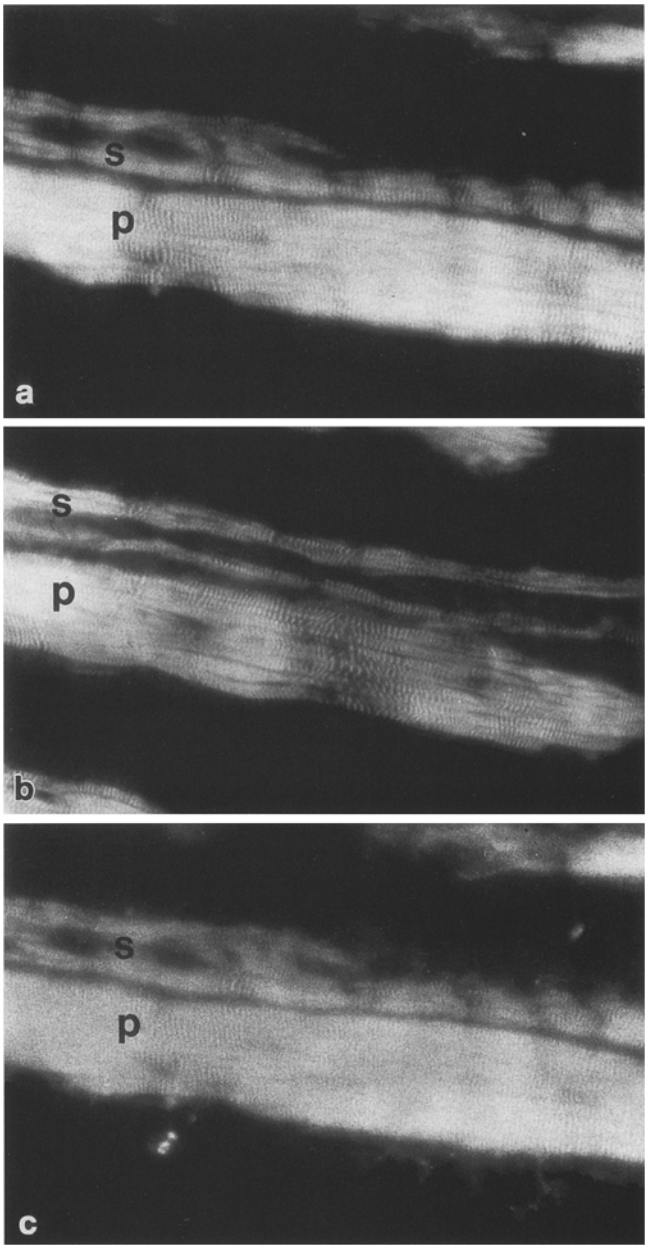

Fig. 3a–c. Serial longitudinal sections of SOL from a newborn rat stained with antibodies against myomesin **a**, M-protein **b** and MM-CK **c**, (**a** and **c** is the same section dual labelled). All three antibodies labelled fibres of both large and small diameter (primary fibres, *p*; secondary fibres, *s*). Note that cross striations can be observed. ×640

(Fig. 6a–c). Furthermore, in the thin cryo-sections additional labelling of anti-MM-CK was observed in the I-band of type 2 fibres (Fig. 6b).

Effects of neonatal denervation

The fibres in the denervated EDL and SOL muscles were considerably smaller than the control non-denervated muscles. No differentiation into fibre types was seen. All fibres in the denervated muscles showed strong myo-

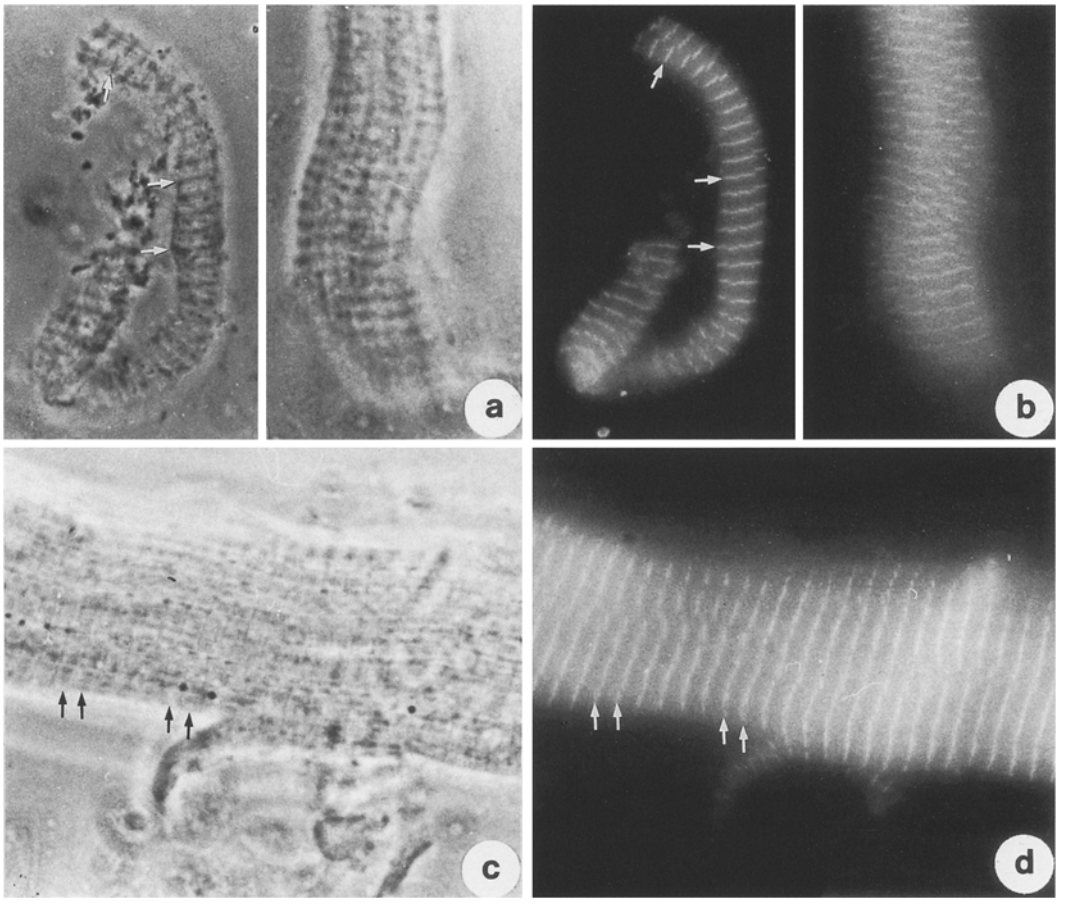

Fig. 4a–d. Washed myofibrils from SOL of newborn **a, b** and four-week-old rats **c, d** stained with anti-MM-CK antibodies. All myofibrils showed M-band staining (**b, d**). Compare the phase contrast (**a, c**) and fluorescence (**b, d**) images at *arrows*. ×900

fibrillar ATPase activity at pH 9.4 and low activity at pH 4.2 (not shown). Furthermore, all muscle fibres were labelled with the antibodies against myomesin (Fig. 7a), M-protein (Fig. 7b) and MM-CK (Fig. 7c).

Discussion

In this study we present the distribution of the myofibrillar M-band proteins in rat skeletal muscles during development. The presence of myomesin in the somites suggests that expression of myomesin is an early event in myofibrillogenesis in mammalia as has been observed in chicken (Grove et al. 1985). Synthesis and incorporation of M-protein in myofibrils is also an early event, however, it takes place later than that of myomesin. MM-CK appears to be incorpororated into the M-band still later. The exact stage when this happens has not been determined because of difficulties with high overall fluorescence of the cytoplasm. However at birth MM-CK is undoubtedly present in the M-band in all fibres together with both myomesin and M-protein. At four weeks of age on the other hand, only a portion of the muscle fibres in SOL and EDL retained expression of all three proteins. At this stage M-protein was no longer detected in type 1 fibres.

From biochemical studies it is known that MM-CK does not become the predominant form of creatine kinase until postnatal development in both rat heart and skeletal muscles (Wegman et al. 1974; Ziter 1974), which would explain the low labelling at the fetal stage. Furthermore, most of the MM-CK is thought to be soluble in the cytoplasm (Wallimann and Eppenberger 1985), which would account for the high overall fluorescence of myofibrils from later stages observed in ordinary cryostat sections after staining with antibodies against MM-CK. At the higher resolution of the semithin cryosections of skeletal muscles of four-week-old rats, high I-band staining, in addition to M-band fluorescence especially in type 2 fibres, was observed. This staining might reflect a further compartmentation of MM-CK which, from a functional point of view, would be analogous to earlier results demonstrating that the bulk of sarcoplasmic glycolytic enzymes are concentrated in this region of the sarcomere (Arnold and Pette 1970) and provide energy for contraction through the creatine phosphate energy shuttle (Savabi et al. 1984; Wallimann and Eppenberger 1985). However, our results and others (Wegmann et al. 1987; Wallimann et al. 1989) are in contrast to a recent paper by Otsu et al. (1989) claiming that "most creatine kinase M is distributed in the A-band".

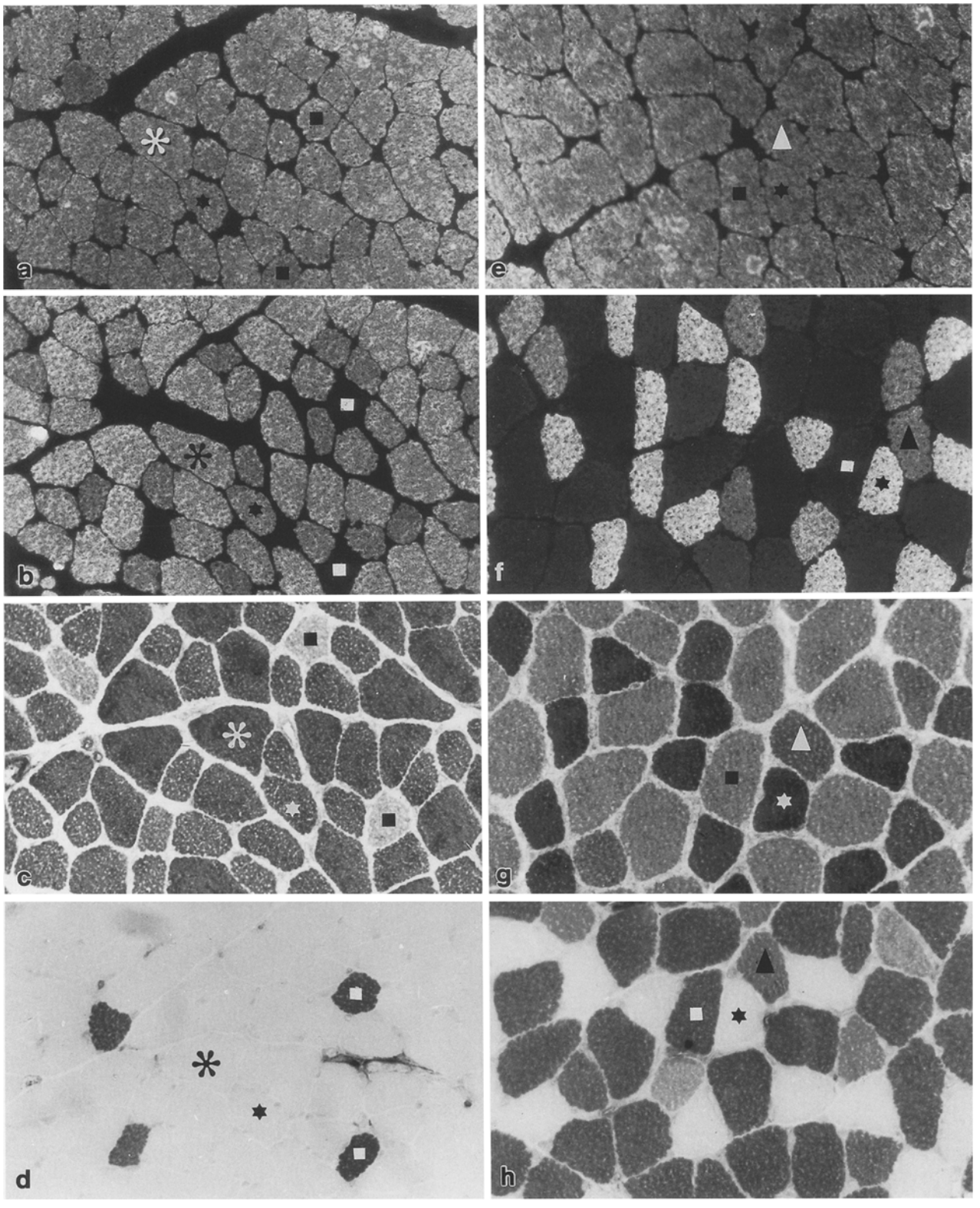

Fig. 5a–h. Serial cross sections of EDL (**a–d**) and SOL (**e–h**) from four-week-old rats stained with anti-myomesin (**a, e**) and anti-M-protein (**b, f**) mAbs and for ATPase at pH 9.4 (**c, g**) and at pH 4.2 (**d, h**). For reference, type 1 (■), type 2A (*), type IIB (*) and type 2C (▲) fibres are indicated. Note that the type 1 fibres lacked M-protein, whereas type 2 fibres showed positive staining in variable degree (**b, f**). × 750

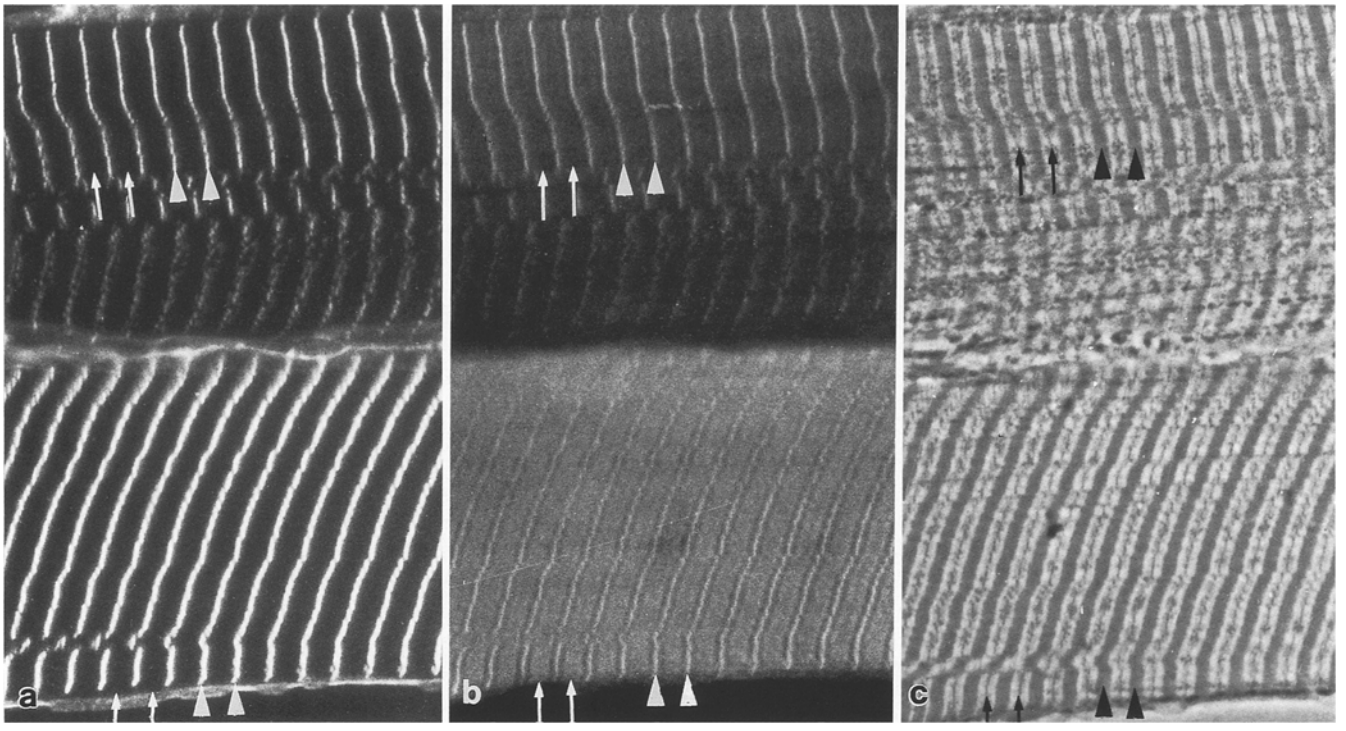

Fig. 6a–c. Longitudinal, semithin (approx. 0.5 μm) cryosection of SOL from a four-week-old rat. Two fibres are seen (*top*, type 1; *bottom*, type 2) dual labelled with antibodies against myomesin **a** and MM-CK **b** and viewed by phase contrast microscopy **c**. Sharp staining of the M-band in the middle of the A-band (*arrowheads*) is seen in both fibres in both **a** and **b**. The type 2 fibre shows also staining for MM-CK in the I-bands **b**. The Z discs are indicated (*arrows*). ×1200

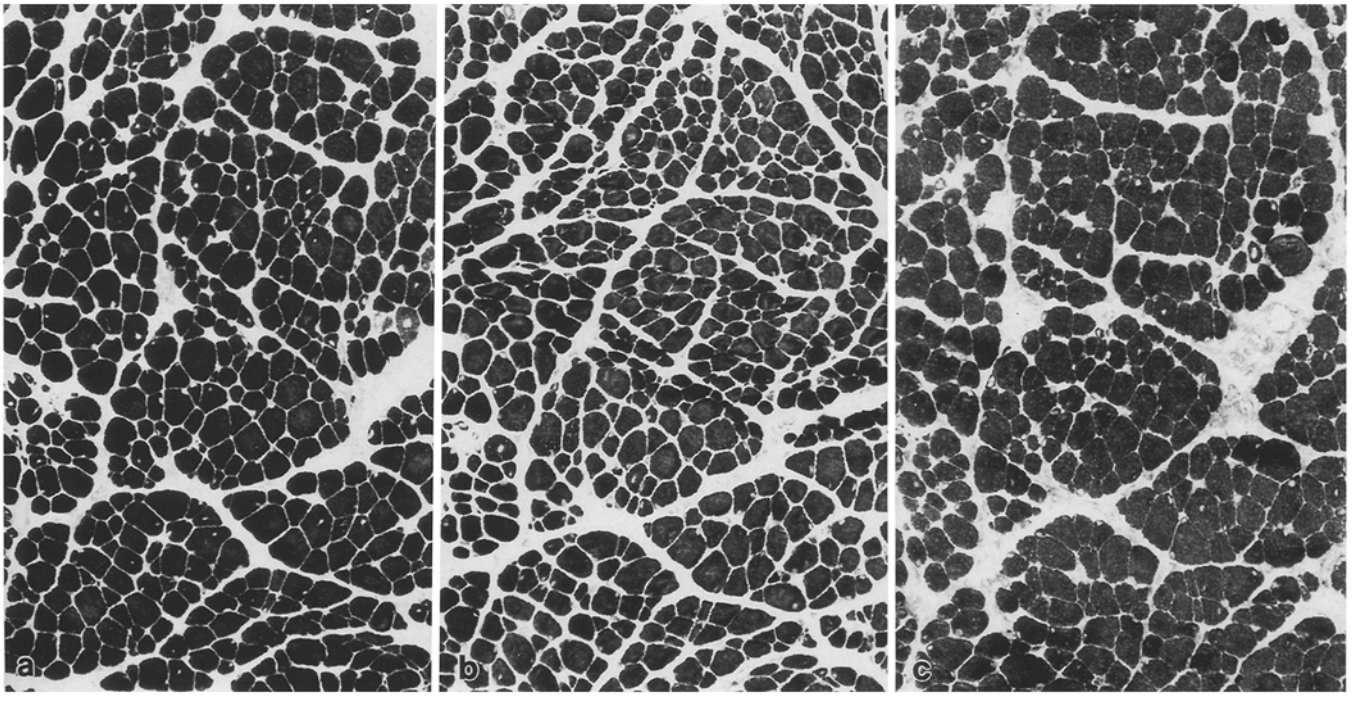

Fig. 7a–c. Serial cross sections of SOL from a four-week-old rat which was denervated at birth. All muscle fibres were a trophic and were labelled with antibodies against myomesin **a**, M-protein **b** and MM-CK **c**. ×640

Table 1. Correlations between M-band composition and ultrastructure

	Myomesin[a]	M-protein[a]	MM-CK[2]	M-band pattern[b]
Skeletal muscle				
Somites (14 days)	+	o/+	o	Not studied
Fetal muscle fibres (16 and 18 days)	+	+	o	Not studied
Newborn				
SOL	+	+	+	Five lines
EDL	+	+	+	Five lines
Four weeks				
SOL type 1	+	o	+	Four lines
SOL type 2	+	+	++	Five lines
EDL type 1	+	o	+	Four lines
EDL type 2	+	+	++	Three or five lines

[a] + present; o not present; +/o present in some fibres
[b] From Carlsson and Thornell, 1987

The present observation that MM-CK is present in the M-band in all skeletal muscle fibers of newborn rats irrespective of fibre diameter is consistent with our previous ultrastructural findings. All primary and secondary generation fibres in both presumptive slow and fast skeletal muscles of newborn rats have dense M-bands in which five cross-striations (M-bridges) interlinking the thick filaments (Carlsson and Thornell 1987). For a comparison of the different data see Table I. Previous observations of a four-line M-band pattern in type 1 fibres and a five- or three-line M-band pattern in type 2A and 2B fibres, respectively (Carlsson and Thornell 1987) might reflect a difference in MM-CK binding at the central bridge line (M1) of the M-band.

As myomesin is present in the M-band at all stages of skeletal muscle development, it can be concluded that myomesin is an integral part of the M-band. Myomesin might thus ensheath the rod portions of the myosin molecules forming the bare zone of the thick filaments as discussed by Wallimann and Eppenberger (1985), Bähler et al. (1985) and Thornell et al. (1986).

M-protein, on the other hand, appears to be related to the specialization of skeletal muscle fibres. The appearance of M-protein showed a positive correlation with the appearance of an electron dense M-band exclusively in fibres shown a five- or a three-line pattern (i.e. all fibres of newborn rats and all type 2 fibres of 4-week-old rats, see Table 1). All type 1 fibres which lacked M-protein have previously been shown to have a four-line M-band pattern with the central M1-line missing. It would thus be tentative to suggest that M-protein make up the M1-line, however, this proposal has to await immunoelectron microscopic studies since in other species like guinea pig we have observed that type 1 fibres lacking M-protein containing a five-line pattern (Thornell et al. 1986; Thornell et al. 1990). Nevertheless, our results show that M-protein itself does not seem to be a prerequisite for the binding of MM-CK to the M-band region since type 1 fibres contain M-band bound MM-CK but no M-protein.

We have observed that the M-protein staining varied among type 2 fibres which can be related to different subpopulations of type 2 fibres. In addition to type 2A, 2B and 2C fibres, another fibre type called type 2X or 2D has recently been identified in rat skeletal muscles (Schiaffino et al. 1989; Termin et al. 1989). The type 2A, 2B and 2D or 2X fibres express specific MHC isoforms whereas the type 2C fibres in SOL muscle from four-week-old rats contain both neonatal and slow myosin (Dhoot 1986; Narusawa et al. 1987) and are undergoing transformation to slow fibres (Kugelberg 1976). From a combined physiological and morphological mapping of rat motor units it is apparent that the structure of the M-band is distinctly different in appearance in slow twitch (type 1) fibres from that in fast twitch (type 2) fibres (Thornell et al. 1987). However, also within the population of fast twitch fibres the structure of the M-band shows some variability. This might reflect the variations in the amount of M-protein present in the different types of fast twitch fibres (Thornell et al. 1987). Our data indicate that during postnatal development of skeletal muscles and apparently in parallel with the differentiation of type 1 fibres synthesis of M-protein is suppressed. A shift in expression from a developmental form of M-protein to a slow isoform would also be compatible with our immunohistochemical results. However, so far there is no evidence for developmental or slow and fast isoforms of myomesin and M-protein in skeletal muscles. Also the epitopes are phylogenetically conserved as our immunoblot data show that the mAbs made against myomesin and M-protein of a chicken fast muscle detected epitopes of the same molecular weights in rat heart and skeletal muscles. Finally, denervation at birth hindered not only normal fibre type maturation as expected but also the disappearance of M-protein in type 1 fibres. Therefore we conclude that the postnatal process of M-protein elimination in type 1 fibres is under neuronal control.

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